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Two binding proteins of the ABC transporter that confers growth of *Bifidobacterium animalis* subsp. *lactis* ATCC27673 on  $\beta$ -mannan possess distinct manno-oligosaccharide binding profiles

Running title: Manno-oligomer bifidobacterial transport proteins

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## Summary

Human gut bifidobacteria rely on ATP-binding cassette (ABC) transporters for oligosaccharide uptake. Multiple oligosaccharide-specific solute binding protein (SBP) genes are occasionally associated with a single ABC transporter, but the significance of this multiplicity remains unclear. Here, we characterize *B/MnBP1* and *B/MnBP2*, the two SBPs associated to the  $\beta$ -manno-oligosaccharide (MnOS) ABC transporter in *Bifidobacterium animalis* subsp. *lactis*. Despite similar overall specificity and preference to mannotriose ( $K_d \approx 80$  nM), affinity of *B/MnBP1* is up to 2570-fold higher for disaccharides than *B/MnBP2*. Structural analysis revealed a substitution of an asparagine that recognizes the mannosyl at position 2 in *B/MnBP1*, by a glycine in *B/MnBP2*, which affects substrate affinity. Both substitution-types occur in bifidobacterial SBPs, but *B/MnBP1*-like variants prevail in human-gut isolates. *B. animalis* subsp. *lactis* ATCC27673 showed growth on gluco- and galactomannans and was able to outcompete a mannan-degrading *Bacteroides ovatus* strain in co-cultures, attesting the efficiency of this ABC uptake system. By contrast, a strain that lacks this transporter failed to grow on mannan. This study highlights SBP diversification as a possible strategy to modulate oligosaccharide uptake preferences of bifidobacterial ABC-transporters during adaptation to specific ecological niches. Efficient metabolism of galactomannan by distinct bifidobacteria, merits evaluating this plant glycan as a potential prebiotic.

## Introduction

The human gut microbiota (HGM) contributes profoundly to maintaining local and systemic health as well as the metabolic homeostasis of the host (Clemente *et al.*, 2012, Zitvogel *et al.*, 2016). Humans lack the digestive enzymes to degrade most complex carbohydrates (El Kaoutari *et al.*, 2013). Therefore, dietary glycans that reach the lower part of the gastrointestinal tract are the prime metabolic resources that modulate the composition and the metabolic output of the HGM (Flint *et al.*, 2015, Koropatkin *et al.*, 2012). The *Bifidobacterium* genus is a prevalent taxon of the microbiota and one of the first colonizers of the human gut (Milani *et al.*, 2017). Members of this genus are proposed to confer improved bowel function, protection from colorectal cancer (in animal models) and inflammatory disorders (O'Callaghan & van Sinderen, 2016). Bifidobacteria ferment carbohydrates to mainly acetate, which is associated with protection against pathogenic infection in mice (Fukuda *et al.*, 2011). Acetate has also beneficial effects on microbial ecology via its conversion by distinct colonic Firmicutes to the short chain fatty acid (SCFA) butyrate (Louis & Flint, 2016) that possesses potent anti-inflammatory effects on the host (Furusawa *et al.*, 2013). The considerable capabilities of bifidobacteria in utilising a diversity of oligo- and polysaccharides (Andersen *et al.*, 2013, Turrone *et al.*, 2017) contributes to their prevalence and abundance in the human (and animals) guts. Notably,

only a few of the carbohydrate active enzymes (CAZymes) (Cantarel *et al.*, 2009) encoded by bifidobacterial genomes are predicted to be extracellular. By contrast, numerous carbohydrate uptake systems (Turroni *et al.*, 2017), especially ATP-binding cassette (ABC) importers (Andersen *et al.*, 2013, Garrido *et al.*, 2011, Parche *et al.*, 2007) are encoded by bifidobacteria. The affinity and specificity of ABC importers is defined largely by the extracellular lipid-anchored solute binding proteins (SBP) in Gram positive bacteria (Ejby *et al.*, 2016, Ejby *et al.*, 2013). After the initial capture by SBPs, oligosaccharide ligands are released into the permease of the transporter, which is formed by two transmembrane domains (TMD) and the translocation is coupled to ATP-hydrolysis by cytoplasmic nucleotide binding domains. The genes encoding the different modules of an ABC importer are typically co-localised on the same locus, consistent with their functional association. Another aspect of the modularity of oligosaccharide-specific ABC importers is the occurrence of more than one SBP per transport system as observed in human gut commensals from *Bifidobacterium* (Turroni *et al.*, 2010, Milani *et al.*, 2016). The functional significance of the multiplicity of SBP is not clear to date.

$\beta$ -Mannans (hereafter referred to as mannans) are abundant plant glycans that play roles as energy reservoirs in seed endosperms, e.g. carob, guar, legumes, coconuts and coffee (Scheller & Ulvskov, 2010), or as structural parts in the hemicellulose cell wall composite (Lundqvist *et al.*, 2002). Structurally, mannans share a common backbone comprising  $\beta$ -(1 $\rightarrow$ 4)-linked mannosyl residues, which may contain glucosyl moieties (glucomannan) or can be galactosyl decorated (galactomannan) at the C6 hydroxyl group in addition to acetylation (Fig. 1A). Besides their relevance as naturally occurring dietary fibers, mannan hydrocolloids, e.g. konjac glucomannan (KGM), Carob bean gum and guar gum (GG) are widely used as food additives (Gallagher *et al.*, 2004, Prajapati *et al.*, 2013). The exposure of humans to mannans is consistent with the fermentation of these fibers in the human gut (Tomlin *et al.*, 1986). Recently, the fermentation of galactomannan has been demonstrated by *Bacteroides ovatus*, which is a model glycan utilising generalist from the dominant Bacteroidetes phylum (Bågenholm *et al.*, 2017). Very recently, the butyrate producing Fimicute *Roseburia intestinalis* has also been shown to be a competitive primary degrader of mannan (La Rosa *et al.*, 2019). Mannan was also reported to stimulate the proliferation of *Bifidobacterium* spp. in humans (Okubo *et al.*, 1994) and mice (Berger *et al.*, 2014, Tomlin *et al.*, 1986). Functional  $\beta$ -mannanases from bifidobacteria have been described (Kulcinskaja *et al.*, 2013, Morrill *et al.*, 2015), but the mannan utilization strategy of members of this genus has not been explored to date.

This study identifies the mannan utilization locus that mediates the uptake and metabolism of  $\beta$ MOS by *Bifidobacterium animalis* subsp. *lactis* ATCC27673 (Loquasto *et al.*, 2013). The functionality of the locus is verified through growth and proteomic analyses. The two ABC importer associated SBPs are structurally and biochemically characterized. This study promotes our understanding of mannan

catabolism by bifidobacteria and oligosaccharide uptake, which is valuable for the design of efficient interventions to manipulate the microbiota based on dietary supplementation to promote human health.

## Results

*Bifidobacterium animalis subsp. lactis ATCC27673 grows efficiently and competitively on soluble mannans*

*B. animalis* subsp. *lactis* ATCC27673 displayed growth on soluble galacto-mannan from Carob and on Konjac gluco-mannan (KGM), while only limited growth was observed on crystalline insoluble ivory nut mannan (IVN) as judged by acidification (Fig. 1B, C). No growth was observed on the highly galactosyl substituted mannan from Guar gum.

To evaluate the competitiveness of this strain in the utilisation of Carob galactomannan, we performed the growth in the presence of model mannan utilising commensal *Bacteroides ovatus* (Bågenholm *et al.*, 2017). The co-growth was performed on the intact polymer and on a hydrolysate thereof prepared by the strictly conserved  $\beta$ -mannanase from *B. animalis* subsp. *lactis* (Morrill *et al.*, 2015). Notably, the growth rate of the co-cultures appeared higher on the intact polymer than the hydrolysate, but the relative proportions of the two strains seemed similar on both substrates (Fig. 1D, E). Interestingly, *B. animalis* subsp. *lactis* efficiently competed with *Ba. ovatus* throughout the exponential phase and outgrew this strain after 12 hours accounting for approximately 85% of the culture (Fig. 1E). The control co-culture on glucose showed a similar growth rate as the carob-hydrolysate, but the ratio between *Ba. ovatus* and *B. lactis* rapidly reached a 50:50 level, which persisted until the end of the culture.

*The mannan utilisation system of B. animalis relies on a secreted endo- $\beta$ -mannanase and an ATP-binding cassette system with two solute binding proteins*

Previously, the extra-cellular endo- $\beta$ -mannanase (BMan5\_8) belonging to the glycoside hydrolase family 5 subfamily 8 (GH5\_8) (Carbohydrate Active Enzymes database at <http://www.cazy.org/>) from *B. animalis* subsp. *lactis* B1-04 (Morrill *et al.*, 2015) was biochemically characterized. This strain possesses a putative mannan utilisation locus (MUL) that encodes intracellular enzymes necessary to degrade  $\beta$ MOS, but lacks the ABC uptake system identified in the present study, consistent with the lack of growth on mannan. By contrast, *B. animalis* subsp. *lactis* ATCC27673, which grows efficiently on soluble mannans, possesses an identical GH5\_8 endo-mannanase and encodes a full ABC transport system in the MUL (Fig. 2A), suggesting that this transporter is the sole uptake system of  $\beta$ MOS in this organism. We analysed mannanase activity on cells growing on carob mannan or glucose to verify the localization of this enzyme. The activity was found largely in culture supernatants ( $0.27 \pm 0.02$  U mL<sup>-1</sup>) with only a minor fraction present in cell pellets ( $0.055 \pm 0.001$  U mL<sup>-1</sup>).

<sup>1</sup>) (Fig. 2B), indicating the secretion of the enzyme to the culture supernatant. The lack of  $\beta$ -mannanase activity from the glucose culture suggested that the activity is induced on mannan and repressed on glucose. The *B. animalis* subsp. *lactis* ATCC27673 MUL encodes a predicted intracellular  $\beta$ -mannosidase (GH2) and a  $\beta$ -glucosidase (GH1) and an ABC transport system (Fig. 2A). Interestingly, this ABC system encodes two putative  $\beta$ MOS-binding proteins, which we have designated *B/MnBP1* and *B/MnBP2*. Both these SBPs together with the GH2  $\beta$ -mannosidase from the same locus were in the top five most upregulated proteins in the proteomic analysis of cells grown on carob galactomannan as compared to the glucose proteome (Table 1, Table S1). This is consistent with a genomic analysis, which revealed that the genes encoding these two SBPs and the associated ABC system are the only transport proteins distinguish the *B. animalis* subsp. *lactis* ATCC27673 strain from the BI-04 counterpart (Elbourne *et al.*, 2017) in support of their crucial role for  $\beta$ MOS uptake. Interestingly, the GH1  $\beta$ -glucosidase in the locus was not upregulated on galactomannan, in contrast to a putative  $\alpha$ -galactosidase of GH36, which may confer the hydrolysis of galactosyl sidechain in internalized galacto- $\beta$ MOS (Table 1, Table S1).

*The two binding proteins B/MnBP1 and B/MnBP2 of the ABC transporter that confers uptake of  $\beta$ -manno-oligosaccharide ( $\beta$ MOS) possess complementary and partially overlapping specificities*

The  $\beta$ MOS binding proteins *B/MnBP1* and *B/MnBP2* were produced recombinantly and characterized. Both proteins had no measurable affinity to monosaccharides (mannose, galactose, glucose or xylose) or non-mannan oligosaccharides (maltotriose, cellotetraose, xylotriose, galactooligosaccharides or raffinose). Thirteen ligands resembling  $\beta$ MOS and mixed gluco-mannooligomers (Fig. S1) were able to bind to at least one of the binding proteins (Fig. 2C, Table 2). The highest affinity was measured for mannotriose with  $K_d$  values of 81 nM and 71 nM for *B/MnBP1* and *B/MnBP2*, respectively. Both proteins displayed similar affinities for  $\beta$ MOS with a degree of polymerisation (DP) 3–6. By contrast, mannobiose was bound with at least 2570-fold lower affinity to *B/MnBP2* compared to mannotriose (Table 2), while the affinity of *B/MnBP1* to the disaccharide was in the same range for the longer  $\beta$ MOS. This differential affinity for disaccharides was also observed on gluco-mannobioses, where *B/MnBP1* showed  $K_d$  values of 4  $\mu$ M and 33  $\mu$ M for mannosyl-glucose (MG) and glucosyl-mannose (GM), respectively, while no binding of these ligands was observed to *B/MnBP2*. A similar trend was observed with galactosyl decorated mannobiose (Table 2).

The thermodynamic parameters and binding stoichiometry for both *B/MnBP1* and *B/MnBP2* towards mannobiose and mannotriose were determined using isothermal titration calorimetry (ITC) and revealed a 1:1 binding driven by a favorable enthalpy change, which was off-set by a large unfavorable binding entropy (Table 3, Fig. S2). The affinity trend and the magnitude of the binding constants were in good agreement with SPR data (Table 2, Table 3), confirming that mannobiose is recognized by  $\sim 5$  kcal mol<sup>-1</sup> additional binding energy by *B/MnBP1* compared to *B/MnBP2*.



*A variant residue between B/MnBP1 and B/MnBP2 contributes to substrate affinity by binding the penultimate reducing end mannosyl unit.*

Crystal structures of *B/MnBP1* in complex with mannobiose, mannotriose and mannopentose as well as the *B/MnBP2* complex with mannotriose were solved to resolutions of 1.6 Å, 2.0 Å, 2.2 Å and 2.0 Å, respectively (Table 4). Both transport proteins adopt a canonical SBP fold (assigned into cluster B according to structural classification (Berntsson *et al.*, 2010)), which comprises two domains of different size joined by a tripartite hinge region with the ligand binding site located at the domain interface (Fig. 3A). The hinge region comprises two short  $\beta$ -strands arranged in an anti-parallel  $\beta$ -sheet spanning the two domains and the loop D380–Y388 and D380–Y386, in *B/MnBP1* and *B/MBP2*, respectively. This hinge loop appeared flexible as judged by the poor electron density in all data sets. Electron densities for the bound  $\beta$ MOS identified the ligand-binding site at the interface between the two domains. The terminal reducing mannosyl unit of the ligand (position 1) is recognized by dense polar contacts by the side chains of W283/281 ( $N^{\epsilon 1}$  - O5), E284/282 ( $O^{\epsilon 2}$  - C1-OH), K287/285 ( $N^{\zeta}$  - C2-OH), S99 ( $O^1$  - C6-OH) and Q307/305 ( $N^{\epsilon 2}$ -C3-OH) in *B/MnBP1/B/MnBP2* (Fig. 3B-D). The mannosyl at position 2 is stabilised by aromatic stacking onto W216/214 and W303/301 in *B/MnBP1/B/MnBP2* and polar interactions between C6-OH and either  $O^{\delta 1}$  or  $O^{\delta 2}$  of D338, whereas the mannosyl at position 3 is stabilized by stacking interactions onto Y339/337 and polar interactions between the C2-OH and  $N^{\epsilon 2}$  N223/221, C3-OH and  $O^1$  G337/335. The most striking difference between *B/MnBP1* and *B/MnBP2* is the substitution of N63 in *B/MnBP1* to a glycine in *B/MnBP2*. This substitution abolishes an extra hydrogen bond between N63  $O^{\delta 1}$  and OH of C2 or C6 (Fig. 3C, D). Another difference is the substitution of D321 *B/MnBP1* to N336 *B/MnBP2*. This substitution is likely to abolish or weaken the H-bond to the C6-OH of the mannosyl at position 2 due to the loss of the charge (distance increase from 2.4 Å to 3.4 Å). Notably, the poor electron density for mannosyl units at positions 4 and to some degree positions 5 was suggestive of lower stringency in the recognition of these residues in *B/MnBP1*. Thus, the binding site is spacious enough to allow the accommodation of two additional mannosyl units (Fig. S3). The space available and the similar affinities for M4 and M5 suggest that this is also valid for *B/MnBP2*.

Two single mutants were constructed to evaluate the role of N63 of *B/MnBP1* in the differential recognition of mannobiose: N63G in *B/MnBP1* (*B/MnBP1\_N63G*) and G61N in *B/MnBP2* (*B/MnBP2\_G61N*). The binding of these mutants to mannobiose and mannotriose was explored with surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) (Table 2, Table 5, Fig. S2). The loss of the asparagine side chain in *B/MnBP1\_N63G* caused a 63-fold and 157 fold-drop in affinity to mannobiose and mannotriose respectively as compared to *B/MnBP1* based on SPR (Table 2, Table 5). By contrast, the *B/MnBP1*-like variant (*B/MnBP2\_G61N*) gained about 100-fold affinity for mannobiose as compared to the wild type protein, with the corresponding gain in affinity for mannotriose being about 60-fold. The kinetics of binding were greatly affected by these mutations,

e.g. the increase of affinity of *B/MnBP2\_G61N* was associated with a marked increase decrease in  $k_{\text{off}}$  (Fig. 4), although the magnitude of the kinetic rate constants could not be determined reliably for both mutants.

## Discussion

Fiber-poor diet is associated with a shift in microbial composition and a weakening of the mucin barrier (Desai *et al.*, 2016, Porter & Martens, 2016) that protects epithelial cells from microbial insult. Restoring microbiota by boosting specific groups considered to be health-promoting is of great importance to combat disease states related to dysbiosis. Increased functional insight into the abilities of the healthy HGM to catabolise non-digestible glycans provides a rational basis for harnessing the therapeutic potential associated with specific bacterial groups via dietary interventions. Oligosaccharide specific ABC importer genes are ubiquitous in bifidobacterial glycan utilization loci and they are common in human gut Clostridia. The occurrence of more than one SBP in association to a single oligosaccharide-specific ABC transporter is observed in genomes of HGM, e.g. *Bifidobacterium longum* subsp. *infantis* (Garrido *et al.*, 2011, Garrido *et al.*, 2015), but the functionality of such systems remains unexplored to our knowledge. Here we describe two SBPs from the manno-oligosaccharide specific ABC systems that confers efficient growth on mannan by *B. animalis* subsp *lactis* ATCC 27673.

### *Basis of specificity for manno-oligosaccharides*

The crystal structures of *B/MnBP1* and *B/MnBP2* provide a structural basis for the specificity for  $\beta$ MnOS. Weaker electron density was observed in the complex structure of *B/MnBP1* with mannopentaose beyond position 3 compared to positions 1–3 (Fig. S3). The specificity for  $\beta$ MnOS is established through the recognition of the axial C2-OH group of the reducing end mannosyl at position 1 by two hydrogen bonds (Fig. 3). Stacking onto W216/W214 (*B/MnBP1/B/MnBP2* numbering) at position 2 restricts space for equatorial C2-OH groups in the glucosyl epimer, which sets preference for the axial C2-OH in mannosyl. Similarly, the C2-OH of the mannosyl at position 3 is within hydrogen bonding distance from the indole nitrogen of W303/301 and the N<sup>62</sup> of Q223/221. The preferential recognition of mannosyl units is in accord with the binding data, which disclosed an affinity drop of 2–3 orders of magnitude for manno-gluco-oligosaccharides as compared to manno-oligosaccharides (Table 2). The tolerance for glucosyl was highest at position 3, whereas the preference for mannosyl was more stringent at positions 2 and 1, as judged by the severe drop or loss of affinity for the GGM trisaccharide (Table 2). This recognition pattern is likely to be important in excluding related non-substrates, e.g. cello-oligosaccharides. Affinity to  $\beta$ MnOS substituted with  $\alpha$ -(1→6)-galactosyl at the reducing end was only about 10-fold lower as compared to the unsubstituted counterparts, which demonstrates tolerance to galactosyl decorations present in galactomannan (Table



2, Fig. S1). Although the binding site of the  $\beta$ MOS is relatively occluded, the C6-OH group of the mannosyl at position 1 is oriented into a solvent exposed cavity, which provides a plausible explanation for the affinity for galactosyl-substituted  $\beta$ MnOS. A similar cavity may allow the accommodation of the same substitution at position 3, whereas sufficient space seems to hinder the binding of  $\beta$ MnOS with adjacent galactosyl substitutions, consistent with the lack of activity of the *B. animalis* subsp. *lactis*  $\beta$ -mannanase on densely galactosylated mannan from Guar gum (Morrill *et al.*, 2015) and the lack of growth on this substrate as opposed to the less substituted galactomannan. The preference of the transport proteins to oligomers of DP 3-4 also correlates to the dominant hydrolysis products of the extracellular mannanase (Morrill *et al.*, 2015).

Comparison of *B/MnBP1/B/MnBP2* to the only structurally and biochemically characterized counterpart from the moderately thermophilic bacterium *Caldanaerobius polysaccharolyticus* (Chekan *et al.*, 2014) reveals pronounced differences: 1) The binding site of *B/MnBP1/B/MnBP2* is occluded, as compared to the more shallow and open binding site of *CpMnBP* (Chekan *et al.*, 2014), 2) Position 1 accommodates the reducing end mannosyl in the bifidobacterial proteins as opposed to binding of the non-reducing end in *CpMnBP*, and 3) the orientation of the binding sites is almost orthogonal (Fig. S4). From the functional point of view, the occlusion of the ligand-binding site and the additional polar and aromatic contacts at position 3 in the bifidobacterial proteins as compared to *CpMnBP* is likely to lower the dissociation rate constant ( $k_{\text{off}}$ ) of the ligands, which may limit the loss of ligands after their capture. Indeed, the measured  $k_{\text{off}}$  values are in the same range as those measured for the arabinoxylooligosaccharide transport protein from the same organism (Ejby *et al.*, 2013), which confers cross-feeding of bifidobacteria on xylan (Rogowski *et al.*, 2015) and co-growth with xylan degraders.

#### *Diversification of uptake preferences by multiple solute binding proteins*

Homologues of *B/MnBP2* or *B/MnBP1* are encoded by the genomes of a variety of bifidobacteria of animal and human faecal isolates *e.g.* from *B. animalis* subsp. *lactis* and *B. adolescentis* clade (Fig. 5). Phylogenetic analysis showed that bifidobacterial  $\beta$ MOS binding proteins could be classified as either *B/MnBP1*- or *B/MnBP2*-like. Depending on the taxonomic group, only a few strains, mainly from *B. animalis* subsp. *lactis* encoded two different copies, whereas most strains encoded a single copy of either *B/MnBP1* or *B/MnBP2*. The former are distinguished by an asparagine, glutamine or a histidine, which are capable of hydrogen bonding in a similar manner to the corresponding N63, whereas *B/MnBP2*-like possess a glycine or more rarely an alanine (Fig. 5). Our mutational analysis provides support for the striking impact of a single substitution on affinity for M2 and M3. The effect of abolishing the N63 polar bond to the mannosyl at position 2 is evident from the considerable increase in  $k_{\text{off}}$  for the *B/MnBP1* N63G variant and from the reciprocal effects for the *B/MnBP2* G61N mutant (Fig. 4). Functional differences were reported between a tandem repeat of two SBPs fused to the transmembrane domains of the GlnPQ ABC of *Lactococcus lactis* that confers the uptake of

glutamine and asparagine in *Lactococcus lactis* (Fulyani *et al.*, 2016). To our knowledge, this is first report of functional difference of separate SBPs associated to the same oligosaccharide specific ABC importer. The expansion of high affinity capture of ligands by the evolution of *B/MnBP1* like proteins is likely to provide an advantage by adapting to the competitive window of targeted glycans to a specific ecological niche. The rationale of maintaining the *B/MnBP2* besides *B/MnBP1* in the *B. animalis* species is unclear, but this maybe an evolutionary transition towards the more common *B/MnBP1*-like in higher animal guts. A similar strategy might have been evolved for targeting related host glycans from human milk or mucin by *B. bifidum* and *B. longum* subsp. *infantis*, which possesses loci that encode up to seven adjacent putative oligosaccharide specific SBP in addition to enzymes that target host glycans (Garrido *et al.*, 2011, Garrido *et al.*, 2015). Notably, both SBPs in the present study were similarly highly upregulated and were in the top five proteins in the differential proteome together with the rest of the components of the ABC transporter and the  $\beta$ -mannosidase required for intracellular polymerization of  $\beta$ MOS (Table 1). The high expression of this uptake system underscores the key contribution of transporters to defining the preference of gut microbiota members to oligosaccharides of distinct size and composition and supporting competitive growth on preferred ligands (Ejby *et al.*, 2016, Leth *et al.*, 2018). The competitiveness of *B. animalis* subs *lactis* is evident from the co-growth competition assays with *Ba ovatus*, which is a model glycan utilisation generalist from Bacteroidetes.

*The mannan utilization system of B. animalis subsp. lactis is atypical compared to other gut microbiota members*

In contrast to abundant dietary glycans such as xylan (Leth *et al.*, 2018, Rogowski *et al.*, 2015, Ejby *et al.*, 2013), the metabolism of mannan has not been addressed in detail. Recent work shows that members of the dominant commensal genus *Bacteroides* possess mannan utilization PULs (polysaccharide utilization loci) that encode the protein apparatus for breakdown of mannans including highly substituted Guar gum (Bågenholm *et al.*, 2017). This strategy relies on one or more outer membrane attached enzymes, in addition to periplasmic enzymes and TonB dependent SusCD oligosaccharide uptake system typically used by *Bacteroides* members. Displaying enzymes at cell surfaces seems to be the most common strategy not only in *Bacteroides*, but also in different Firmicutes (Cockburn *et al.*, 2015, Leth *et al.*, 2018, Møller *et al.*, 2012) and in bifidobacteria (Hinz *et al.*, 2005). Indeed, the modular mannanase of GH26 from *B. adolescentis* has been proposed to be cell-attached (Kulcinskaja *et al.*, 2013). Modular cell-attached carbohydrate active enzymes (CAZymes) may contribute to maintaining the proximity of the substrate close to the cells, thereby minimizing leakage to competitors. The  $\beta$ -mannanase activity was mainly localised to the culture supernatant in *B. animalis* subsp. *lactis* (Fig. 2) and the enzyme was not identified in the cell proteome consistent with its secretion. This is in line with the lack of identifiable cell attachment motifs in the enzyme primary structure, e.g. the sortase mechanism widely employed by

bifidobacteria. The secretion of a CAZyme is highly atypical and has not been previously reported to our knowledge by other human gut microbiota members. The rationale for secretion is unclear, but social behaviour and resource sharing has been proposed amongst bifidobacteria (Milani *et al.*, 2015). The high affinity of the SBP of the  $\beta$ MnOS uptake system seems, however, to enable competitive growth with *Ba. ovatus* (Fig. 1E). On the other hand, *Ba. ovatus* possesses a more elaborate system that allows harvesting energy from more complex glycans, *e.g.* densely branched Guar gum (Bågenholm *et al.*, 2017) as compared to the simpler utilisation system of *B. animalis* subsp. *lactis* that includes intracellular  $\beta$ -mannosidases and  $\beta$ -glucosidases, conserved in the mannan utilisation loci of bifidobacteria, which outlines a plausible model for the utilisation of simple mannans (Fig. 6). A putative  $\alpha$ -galactosidase of GH36 encoded by a different locus was highly upregulated, suggesting that this enzyme confer the removal of galactosyl sidechains in galactomannan, prior to complete depolymerisation by the upregulated GH2  $\beta$ -mannosidase (Fig. 6, Table 1). Besides the dietary content of mannan in natural foods, this glycan is a common food additive and hydrocolloid, which justifies a better understanding of its metabolism by HGM members. This insight on the preference of different taxonomic groups allows taking advantage of these differential preferences to target specific taxa.

## Experimental procedures

**Chemicals** – All chemicals were of analytical grade. The following carbohydrate ligands were used: mannobiose (M2) through to mannohexaose (M6), 6<sup>1</sup>- $\alpha$ -D-galactosyl-mannobiose, and 6<sup>1</sup>- $\alpha$ -D-galactosyl-mannotriose, all with purities >95% were from Megazyme (Wicklow, Ireland). Konjac glucomannan, Ivory nut mannan, Carob galactomannan were also from Megazyme. Glucose, mannose, galactose, xylose, raffinose, maltotriose, and Guar gum were from Sigma-Aldrich (St. Louis, MO, USA).  $\beta$ -Galacto-oligosaccharides ( $[\beta$ -D-Galp-(1,4)]<sub>n=1-5</sub>-D-Glcp) were kindly supplied from DuPont Health and Nutrition Inc. (Brabrand, Denmark). Gluco-mannno-disaccharides,  $\beta$ -Man-(1,4)-Glc and Man $\beta$ -4Glc, were prepared as described elsewhere (Kawahara *et al.*, 2012, Hamura *et al.*, 2013). Gluco-mannotrisaccharides (Man $\beta$ -4Man $\beta$ -4Glc, Man $\beta$ -4Glc $\beta$ -4Glc, and Man $\beta$ -4Glc $\beta$ -4Man) were prepared by reverse phosphorolysis of *Ruminococcus albus*  $\beta$ -1,4-mannooligosaccharide phosphorylase RaMP2 (Kawahara *et al.*, 2012) Reaction mixtures (2.5 mL, 0.56 mg mL<sup>-1</sup> RaMP2, 0.1 M  $\alpha$ -mannose 1-phosphate dicyclohexylamine salt (Liu *et al.*, 2015), 0.2 M acceptor cellobiose (Sigma), Man- $\beta$ -(1 $\rightarrow$ 4)-Glc, or Glc- $\beta$ -(1 $\rightarrow$ 4)-Man) were incubated at 37°C for 24 h, terminated by heating (100°C for 5 min), and the trisaccharides were purified by gel-filtration column chromatography using Bio-Gel P2 (Bio-Rad; 1.6 cm  $\times$  100 cm). The pooled fractions were desalted with Amberlite MB4 (Roam and Haas, Philadelphia, PA), and freeze-dried. Obtained amounts of Man $\beta$ -4Man $\beta$ -4Glc, Man $\beta$ -4Glc $\beta$ -4Glc, and Man $\beta$ -4Glc $\beta$ -4Man were 42 mg, 16 mg, and 15 mg, respectively. All the trisaccharides migrated as single spots in the TLC analysis in 2-

propanol/1-butanol/water (12/3/4, v/v/v). The chemical structures of the trisaccharides were verified by ESI-MS and NMR. ESI-MS was performed using an Executive Mass Spectrometer (Thermo Fisher Scientific). The products gave a signal at 527.16  $m/z$   $[M+Na]^+$ . NMR spectra were recorded in D<sub>2</sub>O (99.9%, Sigma, St. Louis, MO, USA) using Bruker AMX500 (500 MHz, Bruker, Billerica, MA, USA).

**Anaerobic growth and competition assay** – *B. animalis* subsp. *lactis* ATCC 27673 and BI-04 were grown at 37 °C in LAB SEM media (Barrangou *et al.*, 2003) supplemented with 0.5% (w/v) carbohydrate under anaerobic conditions using a Whitley DG250 anaerobic workstation (Don Whitley Scientific Ltd., UK). Growth was monitored by measuring OD<sub>600nm</sub> and pH. The competition assay of *B. animalis* subsp. *lactis* ATCC27673 and *Bacteroides ovatus* ATCC 8483, was performed by growing the strains as above in the presence of 0.5 % (w/v) glucose, Carob galactomannan and a hydrolysate of the latter produced by treatment with a recombinant  $\beta$ -mannanase (2  $\mu$ M B/Man5\_8, 10% (w/v) carob-galactomannan, 37 °C, overnight) from *B. animalis* subsp. *lactis* (Morrill *et al.*, 2015). Equal amount (OD<sub>600nm</sub> units) of exponentially growing cells from both strains were used to inoculate a co-culture to OD<sub>600nm</sub>  $\approx$  0.1 in LAB SEM medium. The co-culture was incubated at 37 °C anaerobically and growth was followed by measuring OD<sub>600</sub> for 25 h. Samples (2 mL) were collected for qPCR analysis at 0, 3.6, 6.8, 12 and 24.6 h. The samples were cooled immediately on ice and cells were collected by centrifugation (8000 x g, 10 min, 4 °C). The pellets were washed with 0.9% (w/v) NaCl and stored at -20 °C until further analysis.

**DNA extraction and quantitative PCR (qPCR)** – DNA was extracted from pellets from 2 mL culture (see above) by a InstaGene™ matrixx (Bio-Rad, California) and quantification was carried out with a NanoDrop 1000 spectrophotometer Thermo Scientific (Saveen Wernerm Aps, Denmark). Relative *Bacteroides* and *Bifidobacterium* were determined by qPCR using SYBR green methodology (Applied Biosystems, USA) and a 7500 Fast Real-Time PCR System (Applied Biosystems, USA). *Bacteroides* was quantified was amplified using primers targeting the 16S rRNA gene (Bergström *et al.*, 2012) and Bifidobacteria were quantified by targeting the elongation factor Tu gene (*tuf*) (Sheu *et al.*, 2010). Samples were analyzed in triplicates in a total volume of 20  $\mu$ L using 10  $\mu$ L Fast SYBR Master Mix, 10 $\mu$ M of each primer (Table S2) and 5 ng template DNA. The PCR conditions were the same for both amplifications and consisted of one cycle of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Finally a dissociation curve analysis was done at 95°C for 15 s, 60°C for 15 s and increasing to 95°C at 2% ramp rate (Bergström *et al.*, 2012). Bacterial counts in the samples at 0, 3.6, 6.8, 12 and 24.6 h were estimated based on standard curves 0.01, 0.1, 1 and 10 ng of DNA harvested from pure cultures of *B. animalis* subsp. *lactis* and *Ba. ovatus*, respectively. The statistical significance was analysed using a one-tailed t-test using Origin.

### *Proteomic analysis sample Preparation*

The differential proteome of *B. animalis* subsp. *lactis* ATCC 27673 cells grown anaerobically in 4 ml LAB SEM medium supplemented with 0.5% (w/v) Carob galactomannan or glucose was analysed. Cells were grown in four biological replicates and harvested in the log phase ( $OD_{600} = 0.6-0.8$ ) by centrifugation (5000 x g, 5 min at 4°C).

Cell pellets were washed twice with ice cold 0.9% (w/v) NaCl before resuspension in lysis buffer. Sample preparation was according to Kulak et al. (Kulak *et al.*, 2014). Briefly, cells were lysed in 60 µl lysis buffer (consisting of 6 M Guanidinium Hydrochloride, 10 mM TCEP, 40 mM CAA, 100 mM Tris pH 8.5). Samples were lysed mechanically using bead beating (3mm beads, 30 Hz for 1 min) (TissueLyser II, Qiagen), boiled (95°C, 5 minutes) after which they were sonicated on high (3x 10 s, at 4°C) in a Bioruptor sonication water bath (Diagenode). Insoluble cell debris were removed by centrifugation (14,000 x g, 10 min at 4°C) and protein concentrations in the supernatants was determined using a Bradford assay (Thermo Fisher Scientific) against a bovine serum albumin standard. Digestion was carried out with 20 µg protein diluted 1:3 with 10% Acetonitrile, 25 mM Tris pH 8.5 and incubated with LysC (MS grade, Wako):protein ratio of 1:50 at 37°C for 4 h. Next, samples were diluted to 1:10 with 10% Acetonitrile, 25 mM Tris pH 8.5 and digested with trypsin (MS grade, Promega):protein ratio of 1:100 and samples were incubated overnight at 37°C. Enzyme activity was quenched by adding 2% (w/v) trifluoroacetic acid (TFA) to a final concentration of 1% (v/v). Prior to mass spectrometry, the peptides were desalted on in-house packed C18 StageTips (Rappsilber *et al.*, 2007). For each sample, 2 discs of C18 material (3M Empore) were packed in a 200 µl tip, and the C18 material activated with 40 µl of Methanol (HPLC grade, Sigma), then 40 µl of 80% (v/v) Acetonitrile, 0.1% (w/v) formic acid. The tips were subsequently equilibrated 2x with 40 µl of 1% (w/v) TFA, 3% (v/v) Acetonitrile, after which the samples were loaded using centrifugation at 1700 g. After washing the tips twice with 100 µl of 0.1% (w/v) formic acid, the peptides were eluted into clean 500 µl Eppendorf tubes using 40% (v/v) Acetonitrile, 0.1% (w/v) formic acid. The eluted peptides were concentrated in an Eppendorf Speedvac, and re-constituted in 1% (w/v) TFA, 2% (v/v) Acetonitrile for Mass Spectrometry (MS) analysis.

### *MS data acquisition and Label-free Quantitative Proteomics Analysis*

Peptides samples were loaded onto a 2 cm C18 trap column (ThermoFisher 164705), connected in-line to a 50 cm C18 reverse-phase analytical column (Thermo EasySpray ES803) using 100% Buffer A (0.1% (w/v) Formic acid in water) at 750 bar, using the Thermo EasyLC 1000 HPLC system. Peptides were eluted over a 260 minute gradient (6–60%, 80% (v/v) acetonitrile, 0.1% (w/v) formic acid, 45°C, 250 nl min<sup>-1</sup>) and the Q-Exactive instrument (Thermo Fisher Scientific) was run in a DD-MS2 top10 method. Full MS spectra were collected at a resolution of 70,000, with an AGC target of 3×10<sup>6</sup> or maximum injection time of 20 ms and a scan range of 300–1750 m/z. The MS2 spectra were



obtained at a resolution of 17,500, with an AGC target value of  $1 \times 10^6$  or maximum injection time of 60 ms, a normalised collision energy of 25 and an intensity threshold of  $1.7 \times 10^4$ . Dynamic exclusion was set to 60 s and ions with a charge state  $<2$  or unknown were excluded. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility. The mass spectrometry data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD013188. The raw files were analysed using Proteome Discoverer 2.2. Label-free quantitation (LFQ) was enabled in the processing and consensus steps, and spectra were matched against the database for *Bifidobacterium animalis* subsp. *lactis* ATCC 27673 (NCBI database accession:CP003941.1). Dynamic modifications were set as Oxidation (M), Deamidation (N,Q) and Acetyl on protein N-termini. Cysteine carbamidomethyl was set as a static modification. All results were filtered to a 1% FDR, and protein quantitation done using the built-in Minora Feature Detector. For analysis of the label-free quantification data, proteins were considered present if at least 2 peptides were identified and abundance ratio were analysed at a significance level of  $p \leq 0.01$ .

*Activity DNS reducing sugar assay* – Mannanase activity assays in culture pellets and supernatants were carried out in a 30 mM sodium phosphate buffer pH 6.5, 150 mM NaCl, 0.005% (w/v) BSA and 0.2% (w/v) Carob galactomannan. The cell pellets were washed twice with 0.9% NaCl and resuspended in 200  $\mu$ L of the same assay buffer. The resuspended cell pellets or the same volume from the corresponding culture supernatants were assayed for 10 minutes in a total volume of 400  $\mu$ L. Reactions were quenched with 600  $\mu$ L 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) and thereafter incubated for 15 min at 90°C followed by  $A_{540\text{nm}}$  measurements using mannose (0–0.8 mM) as a standard. Measurements were done in triplicates and the data are shown as means with standard deviations.

*Expression and purification of the manno-oligosaccharide binding proteins from B. animalis subsp. lactis ATCC27673* – Chromosomal DNA of *Bifidobacterium animalis* subsp. *lactis* ATCC27673 (DSMZ, Braunschweig, Germany) was isolated using standard procedures and used as a template to amplify the gene fragments encoding the manno-oligosaccharide mature predicted SBPs (locus tag numbers, *blac\_00780* and *blac\_00785* and NCBI-proteinID AGW84382 and AGW84383, respectively). The amplicons were cloned within the NcoI and EcoRI restriction sites in pETM11 (a kind gift from Gunter Stier, EMBL, Center for Biochemistry, Heidelberg, Germany) (Dummler et al., 2005) using primers in Table S2 to yield the expression vectors pETM-11\_Blac\_00780 and pETM-11\_Blac\_00785. The single mutants *B/MnBP1\_N63G* and *B/MnBP2\_G61N* were generated using the Quickchange lightning site directed mutagenesis kit (Agilent Technologies, CA, USA) using the primers shown in Table S2. The recombinant mannan binding proteins designated as *B/MnBP1*



(Locus *blac\_00780*), *B/MnBP2* (Locus *blac\_00785*), *B/MnBP1\_N63G* and *B/MnBP2\_G61N*, were produced in *E. coli* BL21(DE3) as N-terminal fusions of a TEV cleavable hexa-histidine tag with the SBP mature polypeptides lacking the native signal peptide (amino acid residues 1–18), but having a 3 amino acid insertion (GAM) between the N-terminus of the protein and the tag cleavage site. Recombinant protein production was performed in 3L baffled flasks containing 1 L of LB broth, supplemented with 50  $\mu\text{g ml}^{-1}$  kanamycin. Cells were grown at 37 °C to an OD<sub>600nm</sub> of 0.5, thereafter the temperature was reduced to 21 °C and expression was induced by addition of IPTG (Isopropyl  $\beta$ -D-1-thiogalactopyranoside) to 100  $\mu\text{M}$  and growth was continued for 16 h. Cells were harvested by centrifugation (20 min, 7000 g, 4 °C), resuspended in binding buffer (10 mM HEPES, 500 mM NaCl, 10 mM imidazole, 10 % glycerol, 0.5 mM DTT (Dithiothreitol) pH=7.4) lysed by a single passage through a high pressure homogenizer and centrifuged (25 min, 30000 g, 4 °C). Clarified lysates were applied onto a 5-mL HisTrap HP (GE Healthcare, Uppsala, Sweden), and purified using a standard protocol recommend by the manufacturer. Eluted pure fractions (SDS-PAGE) were pooled, concentrated (10 kDa cut-off ultrafiltration units; Amicon), applied to a HiLoad Superdex G75 26/60 gel filtration column (GE Healthcare) and eluted with 10 mM MES, pH 6.5 at 1 ml min<sup>-1</sup>. Histidine tags were cleaved off using TEV protease (Tobacco Etch Virus nuclear-inclusion-a endopeptidase) according to (Blommel & Fox, 2007). Cleaved enzymes were recovered after passing through a binding buffer pre-equilibrated 1 mL HisTrap HP column (GE Healthcare). Pure protein samples were concentrated as above and stored at 4 °C until further use.

*Surface plasmon resonance (SPR)* – Affinities of *B/MnBP1* and *B/MnBP2* to the different oligosaccharides were determined using a Biacore T100 (GE Healthcare). The proteins were diluted into 10 mM sodium acetate pH 4.1 to 2.5  $\mu\text{M}$  and immobilized on a CM5 sensor chip using a random amine coupling kit (GE Healthcare) to a density of 2500 response units (RU). Sensograms were recorded and analysed as described (Ejby *et al.*, 2013). Experiments were performed in triplicates in the range 3 nM–10  $\mu\text{M}$  for Mannobiose–mannohexaose and 0.5  $\mu\text{M}$ –1 mM for mannobiose (*B/MnBP2*). To investigate the ligand specificity of *B/MnBP1* and *B/MnBP2*, binding was tested towards 0.005–5 mM mannose, galactose, glucose, xylose, cellotetraose, xylotriose, maltotriose, raffinose and galactooligosaccharides.

*Isothermal titration calorimetry (ITC)* – Binding of mannobiose and mannotriose to *B/MnBP1* *B/MnBP2* and mutants in 10 mM sodium phosphate pH 6.5 was measured at 25 °C using an ITC<sub>200</sub> microcalorimeter (MicroCal). Proteins (14 , 25 or 100  $\mu\text{M}$ ) in the sample cell (200  $\mu\text{l}$ ) were titrated by a first injection of 0.5  $\mu\text{l}$  followed by 18  $\times$  2  $\mu\text{l}$  injections of carbohydrate ligand (140 250 or 3000  $\mu\text{M}$ ) with 120 s between injections and analysed as previously described (Ejby *et al.*, 2013).

*Crystallization and structure determination* – Initial crystallization conditions (0.1 M sodium cacodylate pH 6.5, 0.2M Zn-acetate and 18% w/v PEG-8000 at 278 K) were identified using the Structure crystallization screen 1 and 2 (Molecular Dimensions Inc, FL, USA), using a Mosquito® liquid handling robot (TTP Labtech, UK). *B/MnBP1* and *B/MnBP2* (15 mg ml<sup>-1</sup> in 10 mM MES pH 6.5 and 150 mM NaCl) were co-crystallised with manno-oligosaccharides (1 mM) using vapor diffusion in either sitting or hanging drops and grew for 48 h at 25°C at a 1:1 ratio of protein and reservoir solution. Complexes were grown with a reservoir solution of 0.1 M MES pH 5.5, 0.05 M Zn-acetate and 18% PEG 8000. Crystals were flash frozen directly in liquid nitrogen. Diffraction data were collected at the beamlines PXI and PXIII (SLS, Villigen, Switzerland) and the ID30A-3 beamline (ESRF, Grenoble, France). All datasets were processed with XDS (Kabsch, 2010). The structure of *B/MnBP1*:mannobiose was solved in the orthorhombic space group  $P2_12_12_1$  from a K<sub>2</sub>PtCl<sub>4</sub> (10 mM in crystallization), 15 min soaked crystal, using the single-anomalous diffraction (SAD) method with the experimental phase information obtained from data collected at 11.56 KeV. Experimental phasing, initial model building and refinements were performed in the Phenix software suite. (Adams *et al.*, 2010). Manual structure improvement was done in Coot (Emsley *et al.*, 2010) Ligand molecules were included after the protein parts were built and water molecules were added with Coot or manually. The overall quality of all models was checked using MolProbity (Chen *et al.*, 2010). The data collection and refinement statistics are presented in Table 4. The PyMOL Molecular Graphics System, Version 2.0.6 Schrödinger, LLC was used to explore the models and for rendering.

### *Bioinformatics*

Homologues of the *B/MnBP1* and *B/MnBP2* were retrieved from BLAST searches using these proteins as queries Sequences sharing amino acid sequence identities >55% and sequence coverage >95% were retrieved, aligned and a phylogenetic tree was calculated.

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## Author contributions

MH and MAH have conceived the idea of the study. ME performed the experiments except for the qPCR of the mixed culture, the preparation of the manno-gluco-oligosaccharides, and the proteomics, which were performed by WS, GVZ, and both MJP and ES, respectively. AG and DJS provided help with the X-ray crystallography and the structural analysis. ME, and MAH drafted the first version of the manuscript, and all authors read and contributed to the final manuscript.

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## Tables

Table 1. Differential expressed proteins of *Bifidobacterium animalis* subsp. *lactis* ATCC27673 grown on Carob galactomannan and glucose.

Locus tag	Log <sub>2</sub> -fold <sup>a</sup> change Carob/Glc	SP <sup>b</sup>	CAZy <sup>c</sup>	Putative function <sup>d</sup>
BLAC_03635	6.64	No		ABC transporter, nucleotide binding domain
BLAC_00790	6.52	No		ABC transporter, permease
BLAC_00780	5.21	Yes		ABC transporter, solute-binding protein
BLAC_00800	5.16	No	GH 2	β-Mannosidase
BLAC_00785	4.62	Yes		ABC transporter, solute-binding protein
BLAC_01585	4.38	No		Acetyl-CoA/propionyl-CoA carboxylase
BLAC_00825	3.90	No		N-acylglucosamine 2-epimerase
BLAC_01595	3.80	No		Acyl-carrier-protein S-malonyltransferase
BLAC_01590	3.56	No		Propionyl-CoA carboxylase β-subunit
BLAC_07950	3.21	No	GH 36	α-Galactosidase
BLAC_07905	2.48	No	GH 13	α-Glucosidase
BLAC_05415	2.16	No		Cystathionine beta-lyase
BLAC_06995	2.08	No		Acyl-CoA synthetase
BLAC_07675	1.76	No		ABC transporter, ATP binding protein
BLAC_05995	1.57	No		Transcriptional regulator

<sup>a</sup>Log<sub>2</sub> fold changes of top (log<sub>2</sub> fold change>2) differentially expressed identified in cells grown on Carob galactomannan (Carob) relative to glucose (Glc). <sup>b</sup>Signal peptides (SP) prediction is performed using SignalP v.5.0 (<http://www.cbs.dtu.dk/services/SignalP/>). <sup>c</sup>The Carbohydrate Active enZyme (CAZy) family affiliation. <sup>d</sup>Putative function of the proteins based on BLAST searches against Swiss-Prot.

Table 2. Surface plasmon resonance binding analysis of *B/MnBP1* and *B/MnBP2*

Ligand <sup>a</sup>	<i>B/MnBP1</i>			<i>B/MnBP2</i>		
	$K_d$ (M) <sup>b</sup>	$k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}$ (s <sup>-1</sup> )	$K_d$ (M) <sup>a</sup>	$k_{on}$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}$ (s <sup>-1</sup> )
Mannobiose	$(1.87 \pm 0.12) \times 10^{-7}$	$1.50 \times 10^6$	$2.81 \times 10^{-1}$	$(4.81 \pm 1.30) \times 10^{-4}$		0.08
Mannotriose	$(8.17 \pm 0.03) \times 10^{-8}$	$1.29 \times 10^6$	$1.05 \times 10^{-1}$	$(7.12 \pm 0.01) \times 10^{-8}$	$8.51 \times 10^5$	$6.06 \times 10^{-2}$
Mannotetraose	$(1.27 \pm 0.05) \times 10^{-7}$	$6.06 \times 10^5$	$7.68 \times 10^{-2}$	$(1.28 \pm 0.01) \times 10^{-7}$	$5.13 \times 10^5$	$6.59 \times 10^{-2}$
Mannopentaose	$(1.08 \pm 0.05) \times 10^{-7}$	$5.12 \times 10^5$	$5.53 \times 10^{-2}$	$(1.67 \pm 0.01) \times 10^{-7}$	$3.95 \times 10^5$	$6.57 \times 10^{-2}$
Mannohexaose	$(1.76 \pm 0.03) \times 10^{-6}$	$3.09 \times 10^4$	$5.39 \times 10^{-2}$	$(2.03 \pm 0.04) \times 10^{-6}$		
Galactosyl-mannobiose	$(1.96 \pm 0.09) \times 10^{-6}$			$(4.08 \pm 1.03) \times 10^{-5}$	$2.13 \times 10^3$	$5.98 \times 10^{-2}$
Galactosyl-mannotriose	$(1.13 \pm 0.08) \times 10^{-6}$			$(9.26 \pm 0.04) \times 10^{-7}$	$1.62 \times 10^5$	$1.50 \times 10^{-1}$
MGG	$(1.36 \pm 0.05) \times 10^{-4}$			$(3.07 \pm 0.14) \times 10^{-4}$		
MGM	$(1.81 \pm 0.11) \times 10^{-4}$			$(1.34 \pm 0.32) \times 10^{-4}$		
MMG	$(1.52 \pm 0.47) \times 10^{-5}$			$(8.13 \pm 0.35) \times 10^{-6}$		
GGM				$(1.27 \pm 0.50) \times 10^{-3}$		
GM	$(3.25 \pm 0.25) \times 10^{-5}$					
MG	$(4.40 \pm 1.6) \times 10^{-6}$					

<sup>a</sup>Schematic representation of ligand structures is shown in Fig. S1, the “M” and “G” denote mannosyl and glucosyl units, respectively in gluco-manno-oligomers. <sup>b</sup>Dissociation constants ( $K_d$ ) are means of triplicates with the standard deviations. The binding kinetics are reported for those data sets where the rate constants are within range for reliable quantification.

Table 3. Manno-oligosaccharide binding energetics analysed by isothermal titration calorimetry (ITC).

<b>SBP</b>	<b>Ligand</b>	<b><math>K_d</math> (M)</b>	<b><math>\Delta G^\circ</math> (kcal mol<sup>-1</sup>)</b>	<b><math>\Delta H^\circ</math> (kcal mol<sup>-1</sup>)</b>	<b><math>T\Delta S^\circ</math> (kcal mol<sup>-1</sup>)</b>	<b><math>n</math></b>
<i>B</i> /MnBP1	Mannobiose	$(8.20 \pm 0.8) \times 10^{-7}$	-8.30	$-13.94 \pm 0.24$	-5.64	$0.83 \pm 0.01$
	Mannotriose	$(2.62 \pm 0.2) \times 10^{-7}$	-8.98	$-17.39 \pm 0.13$	-8.41	$0.98 \pm 0.01$
<i>B</i> /MnBP2	Mannobiose <sup>a</sup>	$(3.75 \pm 6.7) \times 10^{-3}$	-3.31	$-53.40 \pm 26.6$	-13.67	$0.32 \pm 13.5$
	Mannotriose	$(5.18 \pm 0.4) \times 10^{-7}$	-8.58	$-33.18 \pm 0.53$	-24.60	$0.83 \pm 0.01$
<i>B</i> /MnBP1_N63G	Mannobiose	$(5.71 \pm 0.5) \times 10^{-6}$	-7.15	$-23.73 \pm 0.12$	-16.58	$0.77 \pm 0.002$
	Mannotriose	$(7.81 \pm 0.9) \times 10^{-6}$	-6.97	$-16.75 \pm 0.22$	-9.78	$0.77 \pm 0.007$
<i>B</i> /MnBP2_G61N	Mannobiose	$(1.07 \pm 0.1) \times 10^{-5}$	-6.77	$-22.96 \pm 0.73$	-16.19	$1.14 \pm 0.017$
	Mannotriose <sup>b</sup>	$(2.64 \pm 2.0) \times 10^{-9}$	-11.71	$-37.83 \pm 0.178$	-26.12	$0.80 \pm 0.016$

<sup>a</sup>Enthalpy and stoichiometry are not reliable due to weak binding and inherent lack of information in the binding sigmoid.

<sup>b</sup>The binding constant is less reliable due to increased affinity (see Fig. S2).

Table 4. Data collection and refinement statistics of the complex structures of *B/MnBP1* and *B/MnBP2*.

	<i>B/MnBP1</i> -M2	<i>B/MnBP1</i> -M3	<i>B/MnBP1</i> -M5	<i>B/MnBP2</i> -M3
PDB code	6I5R	6I5V	6I5W	6FUV
Beamline	SLS PXI	ESRF ID30A-3	ESRF ID30A-3	SLS PXIII
Wavelength (Å)	0.999	0.999	0.999	0.999
Resolution range (Å)	48.8- 1.6 (1.7- 1.6)	42.5-2.0 (2.1- 2.0)	47.4 -2.2 (2.3 -2.2)	40.6-2.0 (2.1- 2.0)
Space group	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	<i>P</i> 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
Unit cell	55.6 74.5 102.4	56.3 75.9 102.3	53.3 73.3 103.5	52.2 64.6 133.3
Unique reflections <sup>a</sup>	56799 (5602)	29709 (2838)	21351 (2049)	28205 (1657)
Multiplicity <sup>a</sup>	11.8 (10.8)	5.1 (5.2)	13.3 (12.8)	3.0 (2.1)
Completeness (%) <sup>a</sup>	99.83 (99.98)	98.82 (97.02)	99.50 (97.57)	90.38 (53.66)
CC <sub>1/2</sub>	0.997 (0.456)	0.999 (0.656)	0.998 (0.445)	0.2751 (1.384)
Mean I/σ(I) <sup>a</sup>	8.57 (1.12)	11.66 (1.33)	10.09 (0.94)	4.45 (0.64)
Wilson B-factor	19.73	34.89	45.46	21.25
<i>R</i> -factor <sup>b</sup>	0.19(0.29)	0.19 (0.30)	0.18 (0.33)	0.18 (0.26)
<i>R</i> -free <sup>b</sup>	0.22 (0.33)	0.26 (0.36)	0.26 (0.37)	0.27 (0.34)
Number of atoms	3912	3696	3547	3963
Macromolecules	3376	3317	3367	3362
Ligands	37	43	99	122
Water	499	336	81	479
Protein residues	428	421	427	427
RMS bonds (Å)	0.011	0.013	0.016	0.015
RMS angles (°)	0.98	1.37	1.02	1.43
Ramachandran favored(%)	97.4	95.2	96	97.4
Ramachandran outliers (%)	0.28	0.24	0.71	0.28
Clash score	5.1	3.65	9.2	7.47
Average B-factor	23.7	44.7	57.3	22.45
Macromolecules	22.8	44.9	56.2	21.2
Ligands	25.8	42.0	71.2	27.8
Water	33.1	43.3	55.6	29.7

<sup>a</sup> Values in the parenthesis are for the highest resolution shell.<sup>b</sup> Values in the parenthesis are for before refinement.

Table 5. Binding of *B/MnBP1* and *B/MnBP2* variants measured by surface plasmon resonance.

Ligand	<i>B/MnBP1_N63G</i>		<i>B/MnBP2_G61N</i>	
	$K_d$ (M) <sup>a</sup>	$K_d$ (relative) <sup>a</sup>	$K_d$ (M)	$K_d$ (relative) <sup>a</sup>
Mannobiose	$(1.18 \pm 0.12) \times 10^{-5}$	$6.3 \times 10^1$	$(4.50 \pm 1.30) \times 10^{-6}$	$9.36 \times 10^{-3}$
Mannotriose	$(1.28 \pm 0.03) \times 10^{-5}$	$1.6 \times 10^2$	$(1.20 \pm 0.01) \times 10^{-9}$	$1.69 \times 10^{-2}$
Mannotetraose	$(1.12 \pm 0.05) \times 10^{-5}$	$8.8 \times 10^1$	Not determined	

<sup>a</sup> $K_d$  of the mutant relative to the wild type protein.

Figure 1. A) Schematic representation of mannan. The backbone consists of  $\beta$ -1 $\rightarrow$ 4 mannosyl (Green) and in glucomannan glucosyl (Blue). The mannosyl residues can be substituted by 1 $\rightarrow$ 6 linked  $\alpha$ -galactosyl units (Yellow). Additionally the C2 and/or C3 positions of the mannosyl units can be acetylated (Ac). B) Growth of *Bifidobacterium animalis* subsp. *lactis* ATCC27673 on Carob galactomannan, konjac glucomannan and on highly substituted galactomannan from Guar gum. C) Acidification of medium during growth of substrates in panel B in addition to insoluble ivory nut mannan is shown. All cultures were biological triplicates with standard deviations. D) Growth of co-cultures of *B. animalis* subsp. *lactis* ATCC27673 and *Bacteriodes ovatus* on carob-galactomannan (Green), hydrolysate of Carob galactomannan with the mannanase *BIGH5\_8* (Morrill *et al.*, 2015) (Red) and a glucose control (Grey). E) The relative proportions of the co-culture in panel D as determined by qPCR at 5 time point. The data are means with standard deviations of biological triplicates. Although the experiment starts with significantly lower fraction of *Bifidobacterium* especially on the Carob mannan ( $P=0.02$ ), the concentration of bifidobacteria is significantly higher during the last two samples ( $P<0.005$ ), whereas the ratio of *Bifidobacterium* is not significantly different from *Bacteriodes* on glucose based on a one tailed *t*-test conducted in Origin.

Figure 2. A) Mannan utilisation loci in *B. animalis* subsp. *lactis* ATCC27673. The locus encodes a  $\beta$ -glucosidase of GH1, an ATP-binding cassette transport system consisting of two solute binding proteins and two adjacent transmembrane domain genes, followed by a  $\beta$ -mannosidase of GH2 and a gene encoding a LacI type transcriptional regulator. The extracellular mannanase *B/Man5\_8* is located on a different locus on the genome and a GH36  $\alpha$ -galactosidase encoded by third locus was highly upregulated during growth on mannan. B)  $\beta$ -mannanase activity of culture supernatants (SN) and cell fractions of cultures of the same strain as A, grown on carob galactomannan (Carob) and glucose (Glc). C) Relative affinities of the *B/MnBP1* and *B/MnBP2* towards mannobiose (man2) through to mannohexaose (man6), galactosyl-mannobiose (galman2), galactosyl-mannotriose (galman3) and mixed gluco-manno-oligosaccharides as determined by surface plasmon resonance. The affinities of *B/MnBP1* N63A and *B/MnBP2* G61N are also indicated. The “M” and “G” designate mannosyl and glucosyl units, respectively and the data is normalized to mannotriose (best ligand for both proteins) and shown in a log<sub>10</sub> scale to account for the large difference in affinity.

Figure 3. A) Ribbon representation of the overall structure of *B/MnBP1* in complex with mannotriose. The SBPs consist of an N-terminal domain (Domain 1, green) and a larger C-terminal domain (Domain 2, brown). The two domains are linked by hinge regions shown in teal. Mannosyl subsites are indicated with number 1–3. B) Close-up of the binding site of *B/MnBP1* in complex with mannobiose in two orientations differing by a 180 ° rotation along the x-axis C) Close-up of the binding site of *B/MnBP1* in complex with mannotriose mannobiose D) Close-up of the binding site of *B/MnBP2* in complex with mannotriose. Two orientations differing by a 180 ° rotation along the x-axis are shown. Difference electron density maps for the ligands were calculated with coefficient  $mF_{obs} - DF_{calc}$  and  $\sigma A$ -weighted and are shown as a light blue mesh.

Figure 4. Ligand binding to *B/MnBP1*, *B/MnBP2* as well as the mutants *B/MnBP1\_N63G* and *B/MnBP2\_G61N* as analysed by surface plasmon resonance. Reference and baseline corrected sensograms and the fits of one binding site models (solid line) depicting the binding of A) mannobiose to *B/MnBP1*. B) mannobiose to *B/MnBP2*. C) mannotriose to *B/MnBP1*. D) mannotriose to *B/MnBP2*. E) mannobiose to *B/MnBP1\_N63G* F) mannobiose to *B/MnBP2\_G61N*, G) mannotriose to *B/MnBP1\_N63G* H) mannotriose to *B/MnBP2\_G61N*.

Figure 5. Phylogenetics of bifidobacterial mannan solute binding proteins. A) The sequence logo conservation of the position N63 in *B/MnBP1* and surrounding residues across *Bifidobacterium* members. The Phylogenetic tree based on the alignment in A) shows that *B/MnBP1*-like proteins possessing a glutamine, asparagine or more commonly a histidine at this position, all potentially capable of hydrogen bonding to the mannosyl at position 2 similarly to *B/MnBP1*. By comparison, *B/MnBP2*-like proteins possess mainly glycine at this position (alanine in a single sequence). The genbank accessions and source strain are indicated as identifiers and strains that possess more than one SBP are colored in red.

Figure 6. A schematic model showing the mannan utilization strategy of *B. animalis* subsp. *lactis* ATCC27673. A modular  $\beta$ -endomannanase comprising a glycoside hydrolase family 5 subfamily 8 (GH5\_8) catalytic module joint to a mannan-binding CBM of family 10 is secreted into the growth medium to confers degradation of mannan to oligomers that are internalized by an ABC uptake system following capture by two associated binding proteins: *B/MnBP1*, which displays high affinity binding to disaccharides and *B/MnBP2*, which preference ligands larger than a disaccharide. Internalised oligosaccharides are likely to be degraded by a  $\beta$ -mannosidase and a  $\beta$ -glucosidase encoded by the same locus.  $\alpha$ -(1→6)-galactosyl substituted manno-oligosaccharides bind with relevant affinity to the transport proteins and are like to be taken up and de-branched by a putative  $\alpha$ -galactosidase of GH36. The mannan utilization model is based on proteomic analysis, and the biochemical data except for the GH1  $\beta$ -glucosidase, which was not observed on this galactomannan,



but which is inferred to catalyse the hydrolysis of the non-reducing glucosyl moieties in galacto-manno-oligosaccharides.













